Reassessment of sst₃ Somatostatin Receptor Expression in Human Normal and Neoplastic Tissues Using the Novel Rabbit Monoclonal Antibody UMB-5

Amelie Lupp a, Falko Nagel a, Christian Doll a, Christoph Röcken b, Matthias Evert c, Christian Mawrin d, Wolfgang Saeger e, Stefan Schulz a

a Department of Pharmacology and Toxicology, University Hospital, Friedrich Schiller University Jena, Jena, b Department of Pathology, Christian Albrechts University, Kiel, c Department of Pathology, Ernst Moritz Arndt University Greifswald, Greifswald, d Department of Neuropathology, Otto von Guericke University Magdeburg, Magdeburg, and e Department of Pathology, Marienkrankenhaus Hamburg, Hamburg, Germany

Abstract

Background: Among the five somatostatin receptors (sst₁–sst₅), the sst₃ receptor displays a distinct pharmacological profile. Like sst₂, the sst₃ receptor efficiently internalizes radiolabeled somatostatin analogs. Unlike sst₂, however, internalized sst₃ receptors are rapidly transferred to lysosomes for degradation. Apart from this, very little is known about the clinical relevance of the sst₃ receptor, which may in part be due to the lack of specific monoclonal sst₃ antibodies.

Methods: Here, we have extensively characterized the novel rabbit monoclonal anti-human sst₃ antibody UMB-5 using transfected cells and receptor-expressing tissues. UMB-5 was then subjected to immunohistochemical staining of a series of 190 formalin-fixed, paraffin-embedded normal and neoplastic human tissues.

Results: Specificity of UMB-5 was demonstrated by detection of a broad band migrating at a molecular weight of 70,000–85,000 in immunoblots from human pituitary. After enzymatic deglycosylation, the size of this band decreased to a molecular weight of 45,000. Tissue immunostaining was completely abolished by pre-adsorption of UMB-5 with its immunizing peptide. In addition, UMB-5 detected distinct cell populations in human tissues like pancreatic islands, anterior pituitary, adrenal cortex, adrenal medulla, and enteric ganglia, similar to that seen with a rabbit polyclonal antibody generated against a different carboxyl-terminal epitope of the sst₃ receptor. In a comparative immunohistochemical study, UMB-5 yielded predominant plasma membrane staining in the majority of pituitary adenomas, pheochromocytomas, and a subset of neuroendocrine tumors. The sst₃ receptor was also present in many glioblastomas, pancreatic, breast, cervix, and ovarian carcinomas.

Conclusion: The rabbit monoclonal antibody UMB-5 may prove of great value in the identification of sst₃-expressing tumors during routine histopathological examinations. Given its unique trafficking properties, these tumors may be potential candidates for sst₃-directed receptor radiotherapy.

Key Words
Neuroendocrine tumors · Somatostatin · Antibody · Somatostatin receptor · Pituitary
Introduction

Somatostatin is a cyclic neuropeptide that inhibits the secretion of a large number of hormones from the anterior pituitary, pancreas, and endocrine cells within the gastrointestinal tract, regulates neurotransmission both in the brain and in the peripheral nervous system, and exerts antiproliferative effects. Additionally, it causes a reduction of gastrointestinal tract motility and gallbladder contractility and plays a regulatory role in the immune system [1–3]. The biological functions of somatostatin are mediated via a family of G-protein-coupled receptors, sst1 to sst5. For sst2, two splice variants have been identified in rodents, the unspliced sst2A and the spliced sst2B, carrying a different carboxyl terminus. In a varying pattern and density, somatostatin receptors are present throughout the body, including brain, pituitary gland, neuroendocrine cells of the gastrointestinal tract and pancreas, thyroid gland, adrenals, and immune system [1–3]. Somatostatin receptors have also been found in many tumors, including pituitary adenomas and neuroendocrine tumors [4, 5], where they mediate inhibitory effects on both hormone secretion and tumor growth [6, 7]. Apart from the tissue distribution, sst receptors differ also in their pharmacological profile for synthetic somatostatin analogs, in their regulation of intracellular signaling pathways, and in their biological functions [6, 8–10]. For the sst3 receptor it has been shown that upon its activation apoptosis can be induced, which displays a unique function among the sst receptor family. Consequently, it has been reported that sst3 stimulation results in an induction of two important pro-apoptotic proteins, p53 and Bax, as well as in a translocation of protein tyrosine phosphatase (PTP) to the plasma membrane [8, 11, 12]. On the other hand, it has been demonstrated that the activated sst3 receptor mediates an inhibition of tumor angiogenesis and growth, via regulation of endothelial nitric oxide synthase (eNOS) and mitogen-activated protein kinase (MAPK) activities [13]. Thus, the sst3 receptor is of considerable interest as a pharmacological target for tumor therapy. Additionally, the sst3 receptor seems to be involved in the control of permeability and biogenesis of epithelial tight junctions and, as a consequence, in the regulation of paracellular conductance [14]. Also with respect to its regulation, the sst3 receptor seems to be different from other members of the sst receptor family. Both human and rat sst3 have been shown to internalize rapidly after agonist stimulation and subsequent phosphorylation at the carboxyl terminal tail through a β-arrestin-clathrin-dependent pathway. In contrast to the sst2A receptor, however, only part of the internalized receptors are redistributed to the plasma membrane after agonist withdrawal. A large proportion of the receptor is sequestered into intracellular clusters of larger vesicles and degraded after multiple ubiquitination, thus leading to profound downregulation of the sst3 receptor after sustained agonist stimulation [10, 15]. This may be the molecular basis for the observation that, after intravenous injection of radiolabeled sst2- or sst3-receptor ligands into mice bearing sst2- or sst3-expressing tumors, with a sst3-directed radioligand a much more pronounced and sustained uptake was observed than with a sst2-directed radioligand [16, 17]. Thus, the sst3 receptor may be of particular interest as a target for receptor radiotherapy of human tumors.

However, in comparison to sst2 and to sst5 receptors, much less is known about the precise tissue distribution, downstream signaling, and function of the sst3 receptor. This may be in part due to the lack of suitable monoclonal antibodies, available in sufficient amounts for immunohistochemical staining of formalin-fixed, paraffin-embedded human tissue samples obtained during routine histopathological preparations.

Recently, we have extensively characterized two novel rabbit monoclonal antibodies against the sst2A receptor and against the human sst5 receptor named UMB-1 and UMB-4, respectively. We have shown that both antibodies selectively detect their cognate receptor in crude membrane extracts from sst receptor-expressing cells and tissues and that they are excellently suited for the assessment of sst2A or sst5 expression in fixed human tissue samples [18–20]. Given the numerous advantages of a rabbit monoclonal antibody compared with the currently available polyclonal anti-human sst3 antisera, we have now generated and thoroughly characterized a rabbit monoclonal antibody directed against the carboxyl-terminal tail of the human sst3 receptor. We have then used the novel antibody UMB-5 to evaluate the prevalence and cellular localization of sst3 in a large series of formalin-fixed, paraffin-embedded human normal and neoplastic tissue samples.

Materials and Methods

Tissue Specimens for Immunohistochemistry

A total of 190 human tumor specimens were obtained from the Departments of Pathology of the Charité Universitätsmedizin Berlin, Ernst Moritz Arndt University Greifswald, Otto von Guericke University Magdeburg, and Marienkrankenhaus Hamburg, Germany. Permission was obtained from the local ethics commit-
Novel Rabbit Monoclonal Anti-Human sst3 Antibody UMB-5

Western Blot Analysis
Human embryonic kidney 293 (HEK-293) cells stably transfected with human sst2, sst3 or sst5 as well as pituitary samples from human autopsy were lysed in detergent buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin, and 10 mg/ml bacitracin). Receptors were enriched using wheat germ lectin agrose beads as described previously [22]. When indicated, somatostatin receptors were deglycosylated using peptide: N-glycosidase F (PNGase F) according to the manufacturer’s instructions (New England BioLabs, Beverly, Mass., USA). Samples were then subjected to 7.5% SDS polyacrylamide gel electrophoresis and immunoblotted onto PVDF membranes. Blots were incubated with the rabbit monoclonal anti-human sst3 antibody UMB-5 (dilution 1:200), followed by a peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA; dilution 1:5,000) and enhanced chemiluminescence detection (Amersham, Braunschweig, Germany).

Immunocytochemistry
Stably sst3-transfected HEK-293 cells were grown on cover-slips overnight and either not exposed or exposed to 1 μM somatostatin-14 (SS-14), 1 μM octreotide or 1 μM pasireotide for 30 min (source of octreotide and pasireotide: Novartis, Basel, Switzerland). They were subsequently fixed and incubated with the rabbit monoclonal anti-human sst3 antibody UMB-5 (dilution 1:100) at 4°C overnight followed by an incubation with Alexa Fluor 488-conjugated secondary antibody (Invitrogen, Karlsruhe, Germany; dilution 1:750). Specimens were mounted as described previously [22] and examined using a Zeiss LSM 510 Meta laser scanning confocal microscope.

Immunohistochemistry
Five-micrometer sections were prepared from paraffin blocks and floated onto positively charged slides. Immunostaining was performed by an indirect peroxidase labeling method as described previously [23]. Briefly, sections were dewaxed, microwaved in 10 mM citric acid (pH 6.0) for 16 min at 600 W, and then incubated either with the rabbit monoclonal anti-human sst3 antibody UMB-5 (dilution 1:20) or with the rabbit polyclonal anti-human sst3 antibody [4823] (concentration 0.1 μg/ml), the rabbit monoclonal anti-sst3, antibody UMB-1 or the rabbit monoclonal anti-human sst3 antibody UMB-4 (dilution 1:10 each) overnight at 4°C. Detection of the primary antibody was performed using a biotinylated anti-rabbit IgG followed by an incubation with peroxidase-conjugated avidin (Vector ABC ‘Elite’ kit, Vector Laboratories, Burlingame, Calif., USA). Binding of the primary antibody was visualized using 3-aminobenzenecarboxylate (AEC) in acetate buffer (BioGenex, San Ramon, Calif., USA). Sections were then rinsed, counterstained with Mayer’s hematoxylin and mounted in Vectamount™ mounting medium (Vector Laboratories). For immunohistochemical controls, UMB-5 was either omitted or adsorbed for 2 h at room temperature with 10 μg/ml of the peptide used for immunizations.

Evaluation of the Staining Patterns
Two independent investigators evaluated all immunohistochemical stainings. In the case of a discrepancy in the scoring between the two investigators, a final decision was achieved by
consensus. All sections were scored by means of the immunoreactivity score (IRS) according to Remmelle and Stegner [24], both noting the intensity of the color as well as the percentage of cells showing a positive staining. The IRS, comprising score values between 0 and 12, was calculated as follows: score [percentage of positive cells] \( \times \) score [intensity of staining] = IRS; score [percentage of positive cells]: no positive cells (0); <10% (1); 10–50% (2); 51–80% (3); >80% (4); score [intensity of staining]: no staining (0); mild (1); moderate (2); strong (3). The classification of the staining as ‘strong’ was based on the intensity of the staining of two positive control locations, pancreatic islets and pheochromocytomas (fig. 1c, d), known from the literature as strongly expressing the sst3 receptor. Only a slight intensity of staining was classified as ‘mild’ (see, e.g., fig. 2: sst3 expression in ACTH adenoma) and the staining intensity between ‘mild’ and ‘strong’ as ‘moderate’.

**Fig. 1.** Immunohistochemical localization of sst3 in human normal and neoplastic tissues using the monoclonal antibody UMB-5. **A** Schematic representation of the sst3 receptor indicating the different carboxyl-terminal epitopes of the rabbit polyclonal anti-human sst3 antibody (4823) and the rabbit monoclonal anti-human sst3 antibody UMB-5. **B** Sections were dewaxed, microwaved in citric acid, and incubated with either the rabbit polyclonal anti-human sst3 antibody (4823) (a, b) or the rabbit monoclonal anti-human sst3 antibody UMB-5 (c–h). Sections were then sequentially treated with biotinylated rabbit IgG and peroxidase-conjugated avidin. Finally, color was developed by incubation in AEC, and sections were counterstained with hematoxylin. Inset For adsorption controls, UMB-5 was incubated with 10 \( \mu \)g/ml of the peptide used for immunizations. Note that both the monoclonal antibody UMB-5 and the polyclonal antibody (4823) yielded similar staining patterns. Arrows = mast cells; arrowheads = enteric ganglion cells. Scale bar (a–h) = 250 \( \mu \)m.
**Results**

**Characterization of the Rabbit Monoclonal Anti-Human sst₃ Antibody UMB-5**

The specificity of UMB-5 was initially monitored using Western blot analysis. When membrane preparations from HEK-293 cells stably transfected with human sst₃, sst₂A, or sst₅ were electrophoretically separated and immunoblotted onto nitrocellulose, UMB-5 revealed a broad band migrating at Mᵋ 70,000–90,000 in sst₃-transfected but not in sst₂A- or sst₅-transfected cells (fig. 3a). UMB-5 was then characterized by immunocytochemical staining of sst₃-transfected cells. When HEK-293 cells stably expressing sst₃ were stained with UMB-5, a distinct immunofluorescence localized at the level of the plasma membrane was detected (fig. 3c). Incubation with somatostatin-14 (SS-14) induced a translocation of the receptor immunoreactivity from the plasma membrane into the cytosol, indicating agonist-induced endocytosis. A similar result was obtained with pasireotide, whereas saturating concentrations of octreotide were not able to stimulate the internalization of sst₃ receptors under otherwise identical conditions (fig. 3c). UMB-5 was also tested for possible cross-reactivity with other proteins present in human tissues. When membrane preparations from pituitaries from human autopsy were

**Fig. 2.** Immunohistochemical localization of sst₂A, sst₃, and sst₅ in human pituitary adenomas. Sections of different types of human pituitary adenomas (non-functioning adenomas (= inactive adenomas; upper panel), ACTH-producing adenomas (middle panel), and GH-producing adenomas (lower panel) were dewaxed, microwaved in citric acid, and incubated with either the rabbit monoclonal anti-sst₂ antibody UMB-1 (left panel), the rabbit monoclonal anti-human sst₃ antibody UMB-5 (middle panel), or the rabbit monoclonal anti-human sst₅ antibody UMB-4 (right panel). Sections were then sequentially treated with biotinylated anti-rabbit IgG and peroxidase-conjugated avidin. Finally, color was developed by incubation in AEC, and sections were counterstained with hematoxylin. Note that, in contrast to sst₂ and sst₅, sst₃ was present in all three types of human adenomas and that the immunostaining for sst₂, sst₃ as well as sst₅ was predominantly localized at the plasma membrane. Scale bar = 500 μm.
electrophoretically separated and blotted onto PVDF membranes, UMB-5 detected a broad band migrating at $M_r$ 72,000–85,000 (fig. 3b, left panel). To test the hypothesis that the bands detected in human pituitary samples would represent the glycosylated sst$_3$ receptor, N-glycosylated proteins were enriched by means of lectin agarose beads and either not deglycosylated (−) or subjected to enzymatic deglycosylation using PNGase F (+). Blots were then immunoblotted with the rabbit monoclonal anti-human sst$_3$ antibody UMB-5 and developed using enhanced chemiluminescence. Immunocytochemistry of HEK-293 cells stably transfected with the human sst$_3$ receptor. Cells were grown on coverslips overnight and either not exposed or exposed to 1 µM somatostatin-14 (SS-14), 1 µM octreotide or 1 µM pasireotide for 30 min, subsequently fixed and immunofluorescently stained with the rabbit monoclonal anti-human sst$_3$ antibody UMB-5. Specimens were then examined by confocal microscopy. Representative results from one of three independent experiments are shown. Scale bar = 20 µm.

Immunoistochemical Localization of sst$_3$ in Human Tumors and in Their Tissues of Origin

The rabbit monoclonal anti-human sst$_3$ antibody UMB-5 was then employed for immunohistochemical stainings of a variety of human normal and neoplastic tissues. A set of tissue sections positively stained for sst$_3$ was also incubated with UMB-5 pre-adsorbed with the respective immunizing peptide, which in all cases led to a complete abolition of the immunostaining (see insert in fig. 1c). For comparison of the staining patterns, adjacent sections were stained with the rabbit monoclonal anti-human sst$_3$ antibody UMB-5 and the rabbit polyclonal anti-human sst$_3$ antibody {4823}, which are directed to
different carboxyl-terminal epitopes of the human sst3 receptor. As depicted in figures 1a–d, both antibodies yielded similar staining patterns in pancreatic islets and pheochromocytomas, two previously described prominent localizations of the sst3 receptor [21]. However, in many cases, the monoclonal antibody UMB-5 yielded a more distinct plasma membrane staining (fig. 1e–h). Prominent sst3 immunoreactivity was found in distinct cell populations of the anterior pituitary, pancreatic islets (fig. 1c), enteric ganglion cells of the intestine (fig. 1e, arrowheads), zona fasciculata and zona reticularis of the adrenal cortex (fig. 1f) as well as in the adrenal medulla. In addition, single strongly stained round-shaped cells were observed, scattered throughout the lamina propria mucosae of the gut (fig. 1e, arrows) or throughout the stroma of various malignant tissues like renal, prostate, breast, cervix, or ovarian tumors, probably representing mast cells.

The presence of sst3 immunoreactivity in the human tumor samples investigated is summarized in table 1. Examples of typical immunostainings are shown in figure 1 and 2. In many positive cases, the immunostaining was predominantly localized at the plasma membrane of the tumor cells. However, in all sst3-expressing tumor entities investigated, not only a marked intranidividual heterogeneity, but also a huge interindividual variability both in the percentage of positive cells and in the intensity of immunostaining was noticed (table 1). Strong sst3 expression was seen in all pituitary adenomas, including non-functioning, ACTH- and GH-producing adenomas (table 1; fig. 2). Interestingly, non-functioning adenomas did not exhibit any detectable or noticeable sst2A or sst5.

<table>
<thead>
<tr>
<th>Tumor type (total number of cases)</th>
<th>sst3 positive cases, n (%)</th>
<th>IRS sst3 mean</th>
<th>min.</th>
<th>max.</th>
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<tr>
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<td>ACTH adenoma (8)</td>
<td>8 (100)</td>
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MTS = Metastases.

Table 1. Presence of sst3 in different human tumor samples as determined by the prevalence and the immunoreactivity score (IRS) according to Remmele and Stegner [24]
expression (fig. 2, top panel). In the majority of the ACTH-producing adenomas, sst\(_{3}\) and sst\(_{5}\) were present (fig. 2, middle panel). In contrast, all GH-producing adenomas expressed sst\(_{3}\) and sst\(_{5}\) and in \(\sim65\%\) of cases sst\(_{2\alpha}\) as well (fig. 2, bottom panel). In addition, all cases of pheochromocytoma exhibited a noticeable sst\(_{3}\) expression (table 1; fig. 1d). About 85% of neuroendocrine tumors and their metastases were sst\(_{3}\) positive, amongst them all insulinomas (table 1; fig. 1g). The sst\(_{3}\) receptor was also detectable in many glioblastomas, pancreatic adenocarcinomas, breast carcinomas (both investigated cases of invasive lobular and solid neuroendocrine carcinomas), cervix carcinomas (all cases of adenocarcinomas and the tumor with neuroendocrine differentiation) as well as ovarian tumors (table 1; fig. 1h). No immunostaining for sst\(_{3}\) was observed in renal clear cell carcinomas, prostate adenocarcinomas, invasive ductal carcinomas of the breast, and squamous cell carcinomas of the cervix uteri (table 1).

**Discussion**

In an effort to provide a monoclonal antibody that can be used for immunohistochemical staining of human sst\(_{3}\) receptors, we extensively characterized the novel rabbit monoclonal anti-human sst\(_{3}\) antibody UMB-5. We show that the very carboxyl-terminal tail of human sst\(_{3}\) can serve as an epitope for the generation of a rabbit monoclonal antibody that effectively stains formalin-fixed, paraffin-embedded human tissues. There are several lines of evidence indicating that UMB-5 specifically detects its targeted receptor and does not cross-react. First, in Western blot analyses, UMB-5 selectively detected its cognate receptor and did not cross-react with other proteins present in crude extracts from sst\(_{2\alpha}\)- or sst\(_{3}\)-transfected cells. Second, enzymatic deglycosylation of the receptor revealed a distinct band at a lower molecular weight, corresponding to the expected molecular weight of the deglycosylated receptor. Third, the antibody yielded a distinct staining of the cell surface of sst\(_{3}\)-transfected cells. As expected from previous investigations with the polyclonal antibody [4823] [25], incubation with somatostatin-14 induced a translocation of the receptor from the plasma membrane into the cytosol, indicating agonist-induced endocytosis. A similar result was obtained with the pan-somatostatin analog pasireotide, whereas octreotide, which displays considerably lower affinity to sst\(_{3}\), was not able to cause internalization of the receptor. Fourth, UMB-5 yielded highly efficient immunostaining of formalin-fixed, paraffin-embedded tissue samples with similar staining patterns as those observed with the polyclonal rabbit anti-human sst\(_{3}\) antibody [4823], but with more distinct plasma membrane staining. Finally, preadsorption of UMB-5 with its immunizing peptide resulted in complete abolition of immunostaining detected in tissue sections.

In normal human tissues, the rabbit monoclonal antibody UMB-5 revealed a strong staining of distinct cell populations in anterior pituitary, pancreatic islands, adrenal cortex and medulla, enteric ganglia and mast cells. The findings with respect to anterior pituitary, pancreas, and adrenal tissue are in line with previous findings obtained with polyclonal antibodies or by mRNA analysis [4, 21, 26–28]. The presence of sst\(_{3}\) in enteric ganglion cells and mast cells (identified by double-labeling experiments showing the presence of mast cell protease-1 in these cells) has been recently demonstrated also by immunohistochemical investigations in the ileum of mice [29, 30].

With respect to neoplastic human tissue, the present study revealed a high prevalence of sst\(_{3}\) in different types of pituitary adenoma, namely in non-functioning adenomas. Similar results have been shown previously by means of receptor autoradiography and mRNA analysis [28, 31–35]. The high prevalence of sst\(_{3}\) in comparison to sst\(_{2}\) and sst\(_{5}\) found in the present investigation in non-functioning adenoma is also in line with clinical findings showing an insensitivity of the majority of the non-functioning adenoma to octreotide or lanreotide therapy and a higher efficacy of pasireotide treatment [33, 36–38]. In the literature, however, also contradictory results on sst receptor expression in non-functioning adenoma have been reported [39, 40]. These discrepancies may be related to the PCR methodology and to the antibodies (monoclonal vs. polyclonal antibodies) used in the latter investigations.

Also the high prevalence of sst\(_{3}\) in pheochromocytoma and neuroendocrine tumors found in our investigations with UMB-5 corresponds well to earlier data obtained with polyclonal antibodies [4, 21, 27, 41]. In contrast to these previous investigations showing primarily cytoplasmic immunoreactivity, the present study revealed a predominance of plasma membrane staining. In accordance with data from the literature obtained with polyclonal antibodies or by means of mRNA analysis [42–46], our investigations revealed also a frequent presence of the sst\(_{3}\) receptor in glioblastoma, pancreatic adenocarcinoma, and in breast, cervix uteri, and ovarian tumors.
In conclusion, we have generated and characterized a novel rabbit monoclonal anti-human sst3 antibody, which enables both immunocytochemistry and immunoblotting experiments as well as the visualization of sst3 receptors in human formalin-fixed, paraffin-embedded tissues during routine histopathological examinations. To our best knowledge, this is the first monoclonal anti-human sst3 antibody yielding a predominance of plasma membrane staining in human tissue samples. In comparison to currently available polyclonal antibodies, the monoclonal antibody UMB-5 has the advantage that it can be produced in unlimited amounts for an unlimited time in almost identical high quality. The sst3 receptor was localized at a high prevalence in non-functioning and in hormonally active pituitary adenomas, in neuroendocrine tumors and also in different other solid neoplasms, which may open up new routes for diagnostic and therapeutic intervention, both pharmacologically and with radiolabeled sst3 receptor ligands.

**Disclosure Statement**

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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